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Biomarkers of immune tolerance in liver transplantation

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Abbreviations: DSA, donor-specific antibody; HCV, hepatitis C virus; HLA, human leukocyte antigen; IL-6, interleukin 6; ISDT, immunosuppressive drug treatment; LT, liver transplant(ation); NK, natural killer; Non-TOL, non-tolerant; PBMC, peripheral blood mononuclear cells; RT-PCR, real time polymerase chain reaction; SOT, spontaneous operational tolerance; TOL, tolerant; Tregs, T regulatory cells.

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Abstract

The liver exhibits intrinsic immune tolerogenic properties that contribute to a unique propensity toward spontaneous acceptance when transplanted, both in animal models and in humans. Thus, in contrast to what happens after transplantation of other solid organs, several years following liver transplantation a significant subset of patients are capable of maintaining normal allograft function with histological integrity in the absence of immunosuppressive drug treatment. Significant efforts have been put into identifying sensitive and specific biomarkers of tolerance in order to stratify liver transplant recipients according to their need for immunosuppressive medication and their likelihood of being able to completely discontinue it. These biomarkers are currently being validated in prospective clinical trials of immunosuppression withdrawal both in Europe and in the United States. These studies have the potential to transform the clinical management of liver transplant recipients by mitigating, at least in part, the burden of lifelong immunosuppression.

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1. Introduction

Liver transplantation (LT) is currently the most successful treatment for end-stage liver failure. Remarkable improvements in short-term allograft and transplant recipient survival have been achieved in the last three decades, due in part to advances in surgical techniques and perioperative care, but also to the introduction of powerful immunosuppressive drug treatments (ISDT) such as calcineurin inhibitors. However, the lifelong use of conventional ISDT, which is required to avoid the risk of rejection and graft loss in the majority of patients, is associated with severe side-effects and increased recipient morbidity and mortality. Amongst complications negatively impacted by chronic ISDT, *de novo* malignancies, infections, chronic renal failure, as well as cardiovascular and metabolic diseases, are the most clinically relevant and altogether constitute the main causes of late LT patient increased mortality [1, 2].

It is now well recognized that the liver exhibits numerous intrinsic immunoregulatory properties that contribute to a unique propensity toward spontaneous acceptance in the context of transplantation and to a far lower risk of graft loss secondary to rejection episodes, as compared with other transplanted organs [3-8]. For example, successful LT can be done without human leukocyte antigen (HLA) matching, across a positive crossmatch, with lower immunosuppressive requirements than other organs, and liver allografts can recover from advanced acute and chronic rejection episodes [4, 9-11]. Moreover, recent data indicate that, using simple clinical, histological and demographic criteria, it is possible to identify a small proportion of liver transplant recipients with approximately 40% chance of being able to successfully discontinue ISDT, depending mainly on recipient age and timing after LT [3, 12-14]. These patients, who can accept the implanted allograft without ISDT for a prolonged period of time, maybe indefinitely, are considered to have developed a state of *spontaneous operational tolerance* (SOT). Although LT recipients meeting these criteria represent a small proportion of the overall transplant population, they have become the focus of intense study. The concrete

clinical opportunity provided by LT patients has fueled the need to identify accurate biomarkers of immune tolerance in order to maximize the benefits that can be derived from ISDT withdrawal. Here we review recent advances in this field.

2. Clinical characteristics of liver transplant recipients achieving spontaneous operational tolerance

In the setting of LT and on the basis of early retrospective and/or single-center studies, a 20% prevalence of SOT was proposed [3, 15-24]. However, this estimation did not take into account the heterogeneity of the inclusion and exclusion criteria employed in the different studies and the fact that, at least in adult recipients, the likelihood of successful ISDT discontinuation is greatly dependent on recipient age and on the time elicited since transplantation [12-14, 25-27]. In the study by Benitez *et al.*, for instance, the effect of time since transplantation was striking, with 13%, 38% and 79% of patients achieving successful ISDT withdrawal depending on whether at inclusion in the study they were <6 years, between 6 and 11 years, and >11 years post-transplant respectively [12]. Taking together the accumulated clinical experience from the early studies and the data derived from the more recent prospective clinical trials, the current agreement is that successful ISDT withdrawal is observed in approximately 40% of recipients when they are selected on the basis of the following criteria: a) >3 years (preferably >6 years) post-transplant; b) no history of autoimmune liver disease; c) no recent episodes of rejection; 4) normal or minimally altered liver histology. These are precisely the patient enrolment criteria being employed in the 2 large multi-centre ISDT withdrawal trials currently underway in the United States and Europe (OPTIMAL trial: NCT02533180 and LIFT trial: NCT02498977 respectively). The results of major published and unpublished biomarker studies in spontaneous operational tolerance following liver transplantation are summarized in Table 1 [12-16, 18, 20-39].

3. Biomarkers of spontaneous operational tolerance in liver transplantation

3.1. Clinical utility of biomarkers of immune tolerance and technical considerations

Because of a significant risk of graft rejection after ISDT withdrawal, there is still a need for prospective identification of individuals who have become operationally tolerant (TOL) to their LT. In this context, biomarker research was developed in order to identify SOT LT recipients prior to ISDT weaning and thus, to reduce radically the risk of rejection. Identification of a reproducible and reliable tolerance «signature» is one of the goals of this research because these biomarkers would substantially benefit the LT population, in modifying the equipoise in favor of ISDT discontinuation in a subgroup of selected patients.

Schematically, the clinical utility of biomarkers of tolerance can be considered in 3 situations:

1. *As a prediction tool*, in guiding patient selection for ISDT withdrawal or tolerance induction protocols;
2. *As a stratification/clinical decision making tool,* in indicating the optimal timing or strategy by which ISDT withdrawal or tolerance induction are most likely to succeed for a patient;
3. *As a monitoring tool,* in serving as an indicator of success or failure of an attempt to establish tolerance.

The ever-expanding *"-omics"* disciplines have provided major opportunities to the identification of biomarkers relevant to transplantation (so called "Transplant*omics*" [40-42]) and, in particular, to the field of LT. The microarray technology, for instance, has been widely employed to evaluate the whole transcriptome of blood and/or liver tissue samples from solid-organ transplant patients. The reproducibility of some of these studies is still an open question, however, given that many solid-organ transplant studies employing high-throughput molecular technologies are small and most likely underpowered. Furthermore, biological interpretations

have been hampered by the fact that there is still a limited understanding on how many biomarkers relate to conventional clinical and immunological outcomes [43].

3.2. Flow cytometric immune cell subset analyses

Early approaches to biomarker discovery in SOT LT recipients involved the analyses of peripheral blood mononuclear cells (PBMC) by flow cytometry. In a Japanese cohort of 12 pediatric SOT LT recipients, Li *et al.* reported an increased frequency of CD4+CD25+ regulatory T cells (Tregs), B cells, and a higher ratio of Vδ1/Vδ2 gamma-delta (γδ) T cells [32]. The latter observation was confirmed in a subsequent study conducted in adult LT recipients [44]. However, Puig-Pey *et al.* demonstrated that alterations in the γδ T cell compartment were not restricted to TOL LT recipients [45]. In fact, most immunosuppressed kidney and LT recipients also displayed an enlarged peripheral blood γδ T cell pool, mainly resulting from an expansion of Vδ1 T cell subset. The increased proportion of Vδ1 T cells was associated with viral infections (cytomegalovirus, hepatitis C infection [HCV]), raising doubts as to the specificity of this marker for tolerance [38, 45]. Finally, Bohne *et al.* conducted a prospective open-label non-controlled ISDT withdrawal trial in which adult HCV-infected stable LT recipients were progressively weaned off immunosuppression [38]. A favorable inclusion biological profile was required, that is with high blood Vδ1/Vδ2 T cell ratio and/or elevated *SLAMF7/KLRF1* transcript levels. The authors demonstrated that blood Vδ1/Vδ2 T cell ratio was useful in the screening of LT recipients for ISDT withdrawal, with an interesting discriminative capacity (82% sensitivity, 53% specificity, 67% positive predictive value, 73% negative predictive value) [38]. They also found that HCV-positive TOL LT recipients were exhibiting an expansion of immune-exhausted HCV-specific CD8+ T lymphocytes [38]. This population of T cells are defined as exhausted because they display a decreased proliferative capacity (through the expression of inhibitory receptors such as PD1 and CTLA4) which can be reversed after inhibitory receptor blockade [38].

Like the above-mentioned Japanese group, several other groups also identified increases in peripheral Tregs in SOT recipients [21, 32, 46]. Pons *et al.* carried out a prospective study to investigate the dynamic profile of the Tregs population in LT recipients during ISDT withdrawal. An increase in the frequency of CD4+CD25+ T cells and in the relative mRNA FoxP3 expression during the ISDT weaning process was observed only in the TOL recipients, and not in those patients who eventually developed rejection [21].

Plasmacytoid dendritic cells (DCs) represent another cell population with regulatory functions. Tolerogenic plasmacytoid DCs have a low capacity for T cell stimulatory functions and a high capacity for inducing tolerogenicity, mainly through downregulation of MHC class II and costimulatory molecules, upregulation of inhibitory factors and secretion of effector molecules and regulatory cytokines (e.g. nitric oxide, IL-10) [47]. In the context of SOT LT recipients, plasmacytoid DCs were observed at higher frequencies in some [48], but not all analyses [44].

Finally, at the graft level, immune cell subset analyses yielded interesting results too. The aforementioned Japanese group expanded their findings in PBMC from pediatric LT recipients to show the γδ T cell «signature» to extend to the graft itself [39]. In separate work, they also described significant accumulations of Tregs in allograft biopsy samples [49].

3.3 Anti-HLA antibodies

The pathogenic role of preexisting or *de novo* anti-HLA donor-specific antibodies (DSAs) is well established after kidney transplantation. In this setting, DSAs represent a risk factor for the development of acute and chronic rejection, as well as graft loss and patient death [50, 51]. In contrast to kidney transplantation, the liver allograft has been traditionally considered resistant to the effects of DSAs. Recently, increasing evidence suggests that DSAs are associated with acute and chronic liver allograft rejection and many other post-transplant complications (e.g. fibrosis progression, ductopenia, biliary complications), which may have detrimental

consequences on allograft and patient outcomes [52-55]. However, these data are mainly issued from single-center retrospective studies and remain contentious.

The potential relevance of DSA monitoring during or after ISDT withdrawal was first proposed by Girnita *et al.* in 2010 [56]. Employing an enzyme-linked immunosorbent assay, the authors observed no DSAs in successfully weaned LT recipients, as compared to recipients under minimal or normal ISDT. These results were not confirmed by Feng *et al.* in a prospective multi-centre drug withdrawal trial in pediatric recipients [13]. In this study, neither HLA mismatch nor presence of DSAs (assessed by single antigen bead assays) were associated with the outcome of ISDT withdrawal [13]. The absence of association between DSAs (detected by enzyme-linked immunoassay, complement-dependent cytotoxicity or flow cytometry) and ISDT withdrawal outcome was also pointed out in the study by Benitez *et al.* [12, 57]. On the other hand, in a Japanese cross-sectional study of 81 pediatric living-donor LT recipients, anti-HLA-DRB1 DSAs (detected by single antigen bead assay), as well as anti-angiotensin II type 1 receptor antibodies, were found to be associated with the presence of long-term progressive graft fibrosis [57].

In conclusion, DSAs remain a topic of debate in the field of LT. DSAs are in fact a reflect of donor sentitization and could therefore represent a potential barrier for the establishment of tolerance. However, in the setting of LT, the uncertainty as to what constitutes the pathogenic determinants of anti-HLA antibodies renders this field difficult to interpret in the context of the prediction of SOT.

3.4. Transcriptional profiling

3.4.1 PBMC gene expression

An alternative approach to cytometric analyses was initiated by Martínez-Llordella *et al.*, using microarray technology gene-expression profiling of PBMC from SOT LT recipients [44]. In a

retrospective, cross-sectional study, 16 SOT LT recipients were compared to 16 non-tolerant (Non-TOL) recipients, and 462 positively and 166 negatively regulated genes were identified. The tolerance-associated molecular «signature» revealed by this study encoded predominantly for natural killer (NK) and γδ T cell receptor-related transcripts and thus, was corroborating preceding cell subset analyses. The following year, the same group published a more robust analysis of a larger cohort of patients and incorporated both training and validation sets, as well as the necessary cross-validation checkpoint procedures, with the aim of validating the predictive capacity of their microarray method [34]. A novel modeling approach, based on the misclassified penalized posterior algorithm, yielded a «signature» comprised of only a small number of genes, containing 2, 6 and 7 genes, respectively and altogether comprising 12 different genes. These «signatures» were shown to be capable of providing high diagnostic accuracy in the identification of tolerance, not only in the group of recipients from whom they were derived but also of an independent validation cohort of 23 subjects.

However, despite a very good diagnostic performance of these blood-based transcriptional biomarker test and the confirmation of the overrepresentation of transcripts preferentially expressed by NK cells in TOL patients, the reproducibility of the test in a multi-centre prospective trial was found to be insufficient, rendering blood-based PBMC molecular «signature» test unreliable to predict the outcome of ISDT withdrawal [36]. This clearly represents an area where there is room for additional research. As an example, in a retrospective cross-sectional study, Li et *al.* amalgamated multi-centric living and deceased donor and pediatric, as well as adult data, gene expression data and identified a 13-gene peripheral blood tolerance «signature» [58]. This «signature» was highly associated with NK cells and proved to have a high predictive accuracy (100% sensitivity, 83% specificity).

γδ T cells, NK cells and tolerance: γδ T cell are “non-conventional” T cells that participate in both innate and adaptive immunity as cytolytic effector cells, but that are also involved in

immunoregulatory responses. The Vδ1 T cell subtype, which is usually not the predominant subpopulation in the peripheral blood of healthy adults, preferentially populates epithelial tissues such as the intestine, where it has been implicated in local immunoregulatory processes, most likely through the killing of either effector T cells, antigen-presenting cells or stressed epithelial cells [44, 59]. Typically, Vδ1 T cell subtype express the activating NK receptors NKG2D and CD160, which contribute to promote their cytolytic effector function [44, 59]. Moreover, genes encoding for γδ T cell and NK receptors are known for their potential to regulate mitosis and cell proliferation. This could corroborate the fact that the above-mentioned Vδ1/Vδ2 T cell ratio is an interesting marker of tolerance, when increased in relation to an expansion of the Vδ1 T cell population.

Differences and similarities between kidney and liver transplant tolerance profiles: Most kidney studies have coincided in the identification of a B cell related transcriptional «signature» in blood, associated with an expansion of B cells with a transitional and/or an IL-10 producing phenotype [60-64]. The kidney and liver blood-derived transcriptional profiles have been directly compared by Lozano et *al.*, which revealed that there were no similarities neither at the transcriptional nor at the flow cytometry level [65].

3.4.2 Liver tissue gene expression

Mechanistic interpretations of the above-mentioned studies were limited by their retrospective design, which could not exclude the confounding effect of pharmacological immunosuppression (i.e. ISDT-free TOL patients were compared with Non-TOL recipients under ISDT), and the lack of simultaneous molecular analyses of allograft tissue. In 2012, the group led by Sánchez-Fueyo published data from a prospective, multi-centre trial of ISDT withdrawal in LT recipients [36]. Of 75 LT recipients completing the trial, 42 (56%) underwent rejection, while 33 (44%) were successfully weaned off ISDT and proved to reach a state of SOT. Both flow cytometric and gene-expression analyses of PBMC confirmed the overrepresentation of NK cells and NK-

related gene sets in TOL recipients. However, the PBMC molecular «signature» lacked reproducibility across the participating centres and could not reliably predict the outcome of ISDT withdrawal. The most accurate and reproducible predictor of ISDT withdrawal outcome proved to be the liver tissue-derived transcriptional profile obtained at baseline. Interestingly, the intragraft expression profile showed no overlap with genes identified from PBMC. In fact, liver biopsy microarrays validated by real-time polymerase chain reaction (RT-PCR) showed that, among the 10 genes showing transcriptional differences of greatest magnitude in relation to tolerance (*TRFC, PEBP1, MIF, CDHR2, SOCS1, IFNG, HAMP, SLC5A12, DAB2, HMOX1)*, there was an overrepresentation of those involved in iron metabolism. These included transferrin receptor 1 *(TRFC),* hepcidin *(HAMP),* and macrophage inhibitory factor *(MIF).* A combination of 5 of the 10 biopsy-derived genes, when measured prior to ISDT weaning, was extremely accurate at discriminating those LT recipients who could successfully withdraw ISDT from those who could not. This predictive «signature» contained the following 5 genes: *SOCS1, TFRC, PEBP1, MIF, CHDR2,* and predicted the outcome of ISDT withdrawal with a sensitivity of 89% and a specificity of 86% (area under the curve 85%, positive predictive value 80%, negative predictive value 92%). This was consistent with the finding that TOL and Non-TOL recipients differed in hepcidin and ferritin serum levels, as well as in hepatocyte iron deposition (higher in LT recipients successfully weaned from ISDT).

Iron metabolism and tolerance: The interplay between iron homeostasis and tolerance is not fully elucidated. It appears to be clear that inflammation, through a mechanism dependant on interleukin 6 (IL-6), induce a rapid increase of hepcidin, which reduces iron export from enterocytes, hepatocytes and macrophages [66-68]. This results in iron accumulation within macrophages and decreased circulating iron levels, which represents an effective defense strategy against extracellular microorganisms that need access to iron to exert their pathogenic effect [66, 68]. In addition to various effects on innate immune responses and inflammation, iron

is also required for the function and differenciation of adaptive immune cells such as T cells. Iron deficiency impairs T cell proliferation *in vitro* [66, 69]. In their recent description utilizing a well characterized mouse model of immunomediated hepatitis, Bonaccorsi-Riani *et al.* showed that iron-hepcidin axis was important in the regulation of intrahepatic lymphocyte activation and function [66].

The assessment of clinical utility and safety of the above-mentioned graft-derived 5-genes «signature» predictive of SOT is the primary objective of a prospective, randomized, multi-centre and international, biomarker-based trial of ISDT withdrawal, currently recruiting patients in Europe (Liver Immunosuppression Free Trial [LIFT]: NCT02498977 and EudraCT number 2014-004557-14) and supported by the UK National Institute of Health Research, as well as the BIODrIM (BIOmarker-Driven Personalized Immunosuppression) EU consortium (Figure 1). Study participants are randomized 1:1 to either non-biomarker-based ISDT weaning (Arm A) or biomarker-based ISDT weaning (Arm B). In Arm A, ISDT is withdrawn regardless of the result of biomarker test. In Arm B, only those found to be “biomarker-positive” (Arm B+, i.e. positive for the validated liver tissue-derived gene expression «signature» indicative of tolerance) are offered ISDT withdrawal; participants with a negative biomarker test result (Arm B-) will remain on their baseline maintenance ISDT. The trial includes the prospective and sequential collection of numerous biological specimens to conduct ancillary mechanistic studies.

Two additional ongoing multi-centre clinical trials in the United States, both supported by the Immune Tolerance Network and also by the National Institute of Allergy and Infectious Diseases and the National Institute of Diabetes and Digestive and Kidney for the former, iWITH (Immunosuppression Withdrawal for Stable Pediatric Liver Transplant Recipients, NCT01638559) and OPTIMAL (Evaluation of Donor Specific Immune Senescence and Exhaustion as Biomarkers of Tolerance Post Liver Transplantation, NCT02533180), will provide further opportunities for biomarker crossvalidation.

3.5. Potential confounding effects of pharmacological immunosuppression on tolerance biomarker profiles

The capacity of routinely employed immunosuppressants to influence tolerance-related cellular and transcriptional biomarkers has recently been highlighted by Rebollo-Mesa *et al.* in the setting of kidney transplantation [70]. While this has not been explored in such detail in LT, it could have influenced the results of some of the reported studies outlined above, which employed a case-control cross-sectional design. A clear example of this risk is the observation by Bohne *et al.* that TOL recipients exhibit a higher number of circulating CD4+CD25+Foxp3+ regulatory T cells (Tregs) than non-TOL patients but only at the end of the drug withdrawal protocol (once tolerant patients no longer receive calcineurin inhibitors) and not before weaning is initiated [36]. The risk of immunosuppressants modifying biomarker profiles derived from prospective ISDT withdrawal trials is significantly lower than in case-control studies but cannot be completely excluded, particularly in what regards blood-derived biomarkers. For this reason, it is imperative to specifically explore the potential influence of these medications, as well as other treatments. To date this has only been reported for the liver tissue transcriptional «signature» described by Bohne *et al.,* which was found to predict the outcome of ISDT withdrawal independently from the type of immunosuppression administered at the initiation of the trial.

4. Conclusions

Long-term outcomes in LT are hampered by the burden of lifelong ISDT. ISDT withdrawal or minimization could be a logical solution to this problem. This goal seems to be more feasible in the context of LT than in other transplantation settings, as the liver allograft exhibits a relatively privileged immune tolerance status. In order to identify patients suitable for a safe withdrawal or minimization of these medications, substantial effort has been devoted to identify biomarkers with high sensitivity and specificity. Based on these pioneering studies, the first biomarker-led,

prospective ISDT withdrawal trials are underway and promise further progress in tolerance biomarker research. To date most studies have provided descriptive immunophenotyping information derived from multi-parameter flow cytometry and gene expression approaches, and there has been very little emphasis on elucidating the characteristics of donor-specific functional responses. A thorough mechanistic understanding of the TOL phenotype will require incorporation of such strategies into the current biomarker pipelines.

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Year of publication	Authors	Total number of LT patients analysed	Number of TOL LT patients	Number of non-TOL LT patients	Number of STA LT patients	Biomarkers
1998 [16]	Devlin *et al.*	18	5	12	0	• Donor-derived microchimerism (-)
1998 [28]	Wong T *et al.*	37	6	24	0	• Liver tissue immunostaining studies
2001 [30]	Takatsuki *et al.*	23	23	0	0	• Mixed lymphocyte reaction • Intra-graft cytokine profiles
2003 [31]	Mazariegos *et al.*	40	6	34*	0	• Plasmacytoid DC/monocytoid DC ratio (+)
2003 [22]	Pons *et al.*	9	3	6	0	• Endothelial cell chimerism (-)
2008 [21]	Pons *et al.*	12	5	7	0	• Peripheral blood CD4+CD25^{high} T cells (+) • PBMC gene expression (+)
2004 [32]	Li *et al.*	31	12	0	19	• PBMC subsets: CD4+CD25^{high} T cells (+), B cells (+), Vδ1/Vδ2 gamma-delta T cell ratio (+)
2008 [34]	Martinez-Llordella *et al.*	80	28	33	19	• Peripheral blood gene expression
2011 [35]	Castellaneta *et al.*	78	26	28*	24	• HLA-G expression on circulating monocytoid DC (+)
2012 [13]	Feng *et al.*	20	12	8	0	• Anti-HLA antibodies • Liver biopsy C4d score
2013 [12] 2012 [36] 2016 [37]	Benitez *et al.* Bohne *et al.* Taubert *et al.*	102	41	61	0	• Anti-HLA antibodies • PBMC and liver tissue gene expression • Blood cell immunophenotyping • Iron status parameters • Liver tissue immunofluorescence
2013 [14]	de la Garza *et al.*	24	15	9	0	• Stimulation index of circulating lymphocytes following phytohemagglutinin stimulation
2013 [39]	Zhao *et al.*	34	9	17	0	• Intragraft Vδ1/Vδ2 gamma-delta T cell ratio (+) • CDR3 sequencing of the δ chain of Vδ1 cells
2014 [38]	Bohne *et al.*	34	17	15	0	• Blood Vδ1/Vδ2 gamma-delta T cell ratio (+) • Blood SLAMF7/KLRF4 gene expression (+) • Liver tissue gene expression • Anti-HCV Elispot T cell responses • Exhaustion markers in circulating HCV-specific CD8+ T cells
Unpublished [27]	Feng *et al.*	88	33	55	0	• Anti-HLA antibody subclasses; auto-antibodies • Liver biopsy C4d score • Liver tissue multi-parameter immunofluorescence • Liver tissue gene expression

Table 1. Biomarker studies in spontaneous operational tolerance following liver transplantation.

*Patients with unknown status of tolerance (e.g. patients undergoing prospective ISDT weaning, patients with follow-up shorter than 1 year post-ISDT withdrawal) are also included in this category.

(+), positive association with a successful ISDT withdrawal
(-), negative or no association with a successful ISDT withdrawal

CDR3, complementarity-determining region 3; HCV, hepatitis C virus; HLA, human leukocyte antigen; ISDT, immunosuppressive drug treatment; LT, liver transplant; N/A, not applicable; Non-TOL, non-tolerant; PBMC, peripheral blood mononuclear cell; STA, stable LT recipients receiving maintenance immunosuppressive drugs; TOL, tolerant.

Figure 1. Liver Immunosuppression Free Trial (LIFT) flow diagram (NCT02498977).

After a clinical eligibility screening and a confirmation histological eligibility screening through a baseline liver biopsy (see www.clinicaltrial.gov for detailed inclusion and exclusion criteria), consenting study participants are randomized 1:1 to either non-biomarker-based immunosuppressive drug treatment (ISDT) weaning (Arm A) or biomarker-based ISDT weaning (Arm B).

The biomarker test used in this trial is a validated liver graft-derived 5-genes signature which is highly predictive of spontaneous operational tolerance, before ISDT withdrawal. In Arm A, ISDT is withdrawn regardless of the result of biomarker test. In Arm B, only those found to be "biomarker-positive" (Arm B+) are offered ISDT withdrawal; participants with a negative biomarker test result (Arm B-) will remain on their baseline maintenance ISDT.

ISDT, immunosuppressive drug treatment; PIs, principal investigators.